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PURIFICATION AND CRYSTALLIZATION OF 15-LIPOXYGENASE FROM RABBIT RETICULOCYTES

David L. Sloane,[1] Michelle F. Browner,[2,3] Zbigniew Dauter,[5]
Keith Wilson,[5] Robert J. Fletterick,[2,3] and Elliott Sigal[1,4,*]

[1]Cardiovascular Research Institute (CVRI) and
[2]Program of Excellence in Molecular Biology, CVRI, and
Departments of [3]Biochemistry and Biophysics and [4]Medicine
University of California, San Francisco, CA 94143

[5]EMBL Hamburg Outstation, Germany

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We report a new purification of rabbit reticulocyte 15-lipoxygenase that has resulted in the first crystallization of a mammalian lipoxygenase. The enzyme was purified to homogeneity (greater than 98% pure by SDS-PAGE) using high pressure liquid chromatography on hydrophobic-interaction, hydroxyapatite and cation-exchange columns. Crystals were grown by the vapor diffusion method from concentrated solutions of the protein in sodium phosphate buffer, pH 7.0. The hexagonal, rod-shaped crystals were on average 0.09 mm x 0.09 mm x 0.4 mm, with approximate unit cell dimensions of a=b=260 Å, c=145 Å. The crystals diffract to 5 Å resolution.

The lipoxygenases comprise a family of enzymes which catalyze the stereospecific dioxygenation of polyunsaturated fatty acids with a 1,4-cis-pentadiene structure. The enzymes have varied regional specificity and are designated 5-, 12-, or 15-lipoxygenase based on their ability to dioxygenate the carbon 5, 12, or 15 of arachidonic acid. Lipoxygenase metabolites of arachidonic acid are potent biological agents implicated in a variety of cellular responses. For example, 5-lipoxygenase initiates the biosynthesis of leukotrienes, which are critical mediators of the inflammatory response seen in asthma, allergy, and arthritis [1, 2]. The 12-lipoxygenase is preferentially expressed in human platelets and may contribute to mitogenic responses and to smooth muscle cell migration in the circulatory system [3-5]. Several biological actions have been proposed for the 15-lipoxygenase based on its tissue-specific expression and its ability to peroxidize phospholipids as well as free fatty acids [6]. In the developing red cell, 15-lipoxygenase appears to contribute to cellular differentiation via lipid peroxidation of intracellular membranes [7, 8]. In

*To whom correspondence should be addressed: Cardiovascular Research Institute, Box 0911, University of California, San Francisco, CA 94143.

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vascular tissue, 15-lipoxygenase is implicated in the oxidative modification of low-density lipoprotein, a critical step in the formation of atherosclerotic lesions [9, 10, 11]. In human lungs, 15-lipoxygenase has been detected in airway epithelium, where it may generate inflammatory mediators in response to external stimuli [12, 13]. Finally, lipoxygenases are also present in plants, and some evidence suggests that they may have a role in the biosynthesis of pest-resistant compounds [14, 15]. In particular, the biochemical properties of soybean lipoxygenase have been investigated in detail [16].

Lipoxygenase protein has been purified from both plant and animal tissues [16-20]. The cDNAs encoding the soybean lipoxygenase, and the mammalian 5-, 15-, and 12-lipoxygenases have been cloned and sequenced [21-29]. The mammalian 15-lipoxygenase is a 75,000-dalton protein containing a non-heme iron cofactor [7] postulated to be bound by histidines [22, 26, 30]. Five conserved histidines are found in the primary structures of all lipoxygenases examined to date, but knowledge of the structural requirements for iron-binding remains incomplete. Kinetic analysis of the reticulocyte 15-lipoxygenase has led to the suggestion that the enzyme is inactivated by a suicidal mechanism [31], but the exact nature of this inactivation is not clear. The amino acid residues responsible for catalysis, substrate binding, and substrate specificity are also unknown. No three-dimensional structure determination of any lipoxygenase has been reported. Clearly, an atomic resolution structure of this class of enzymes is of interest for both mechanistic studies and for designing therapeutic agents that may be used to control the production of the mediators that derive from lipoxygenase activity.

Soybean lipoxygenase isozyme-I was first crystallized by Theorell [32] and most recently by Steczko et al. [33, 34]. Stallings et al. [35] have reported preliminary x-ray data on crystals of soybean lipoxygenase isozyme-II. To pursue structural studies on mammalian 15-lipoxygenase, we have focused on the rabbit reticulocyte 15-lipoxygenase which can be induced *in vivo* by experimental models of anemia [7]. Although this source is known to yield reagent quantities of purified enzyme, the purification method previously reported did not lead to protein crystals. In this communication we describe a new, rapid purification procedure for 15-lipoxygenase from rabbit reticulocytes that results in 15-lipoxygenase that can be crystallized.

EXPERIMENTAL PROCEDURES

The protocols for bleeding anemia, cell lysis, and ammonium sulfate precipitation have been described previously [7]. All purification steps were carried out at 4°C or on ice. At each step of the purification, oxygen was purged from all buffers by bubbling argon through the buffers. The method of Bradford [36] was used for determining protein concentrations. For a typical large-scale purification, 240 ml of whole blood enriched in reticulocytes (40% of the red cell mass) was washed, lysed and precipitated with ammonium sulfate. The 55% ammonium sulfate precipitate was dissolved in distilled, deionized water and was dialyzed for 16 hours against 40 volumes of MES-1 (20 mM MES, pH 7.0, 0.1 mM EDTA, 20 μM ferrous ammonium sulfate, 1 mM DTT, 7.5% saturated ammonium sulfate). During dialysis, a fine precipitate formed which was removed by centrifugation (4,000 x g, 10 minutes) and

filtration through a 0.22-µm Millex-GV filter unit (Millipore, Bedford, MA). The protease inhibitors leupeptin (0.5 µg/ml), pepstatin (0.7 µg/ml), and PMSF (0.2 mM) (Boehringer Mannheim Biochemicals, Indianapolis, IN) were present throughout the column purifications. The ammonium sulfate sample was applied at 5 ml/min to a 150- x 21.5-mm hydrophobic interaction column (Bio-Gel TSK Phenyl-5-PW, Bio-Rad, Richmond, CA) equilibrated in MES-1. The column was developed on a Waters 840 chromatography system by a linearly decreasing ammonium sulfate gradient until the ammonium sulfate concentration was zero. The fractions containing lipoxygenase activity eluted at approximately 0.4% saturated ammonium sulfate. The active fractions were pooled and concentrated by ultrafiltration (YM-30 membrane, Amicon, Danvers, MA). Potassium phosphate buffer, pH 7.0, was added to a final concentration of 50 mM. This material was next applied at 0.5 ml/min to a 4- x 50-mm high-pressure hydroxyapatite column (Bio-Gel HPHT, Bio-Rad, Richmond, CA) equilibrated in 50 mM KPO_4, pH 7.0, 20 µM ferrous ammonium sulfate, 1 mM DTT. The column was eluted with 250 mM KPO_4, pH 7.0, 20 µM ferrous ammonium sulfate, 1 mM DTT. The peak protein fractions were pooled, concentrated to 4 ml by ultrafiltration, and dialyzed for 16 hours against 2 liters MES-2 (20 mM MES, pH 6.0, 1 mM EDTA, 20 µM ferrous ammonium sulfate, 1 mM DTT). For the final purification step, the sample was applied at 0.5 ml/min to a 5- x 50-mm Mono-S cation exchange column (HR 5/5, Pharmacia, Piscataway, NJ) equilibrated with MES-2 buffer. After the sample had been completely loaded, the flow rate was increased to 1 ml/min over 10 minutes, and the column developed with a linear gradient of NaCl (0-600 mM) in MES-2. The peak fractions eluted at approximately 450 mM NaCl and were pooled, concentrated, and dialyzed against MES-2. In some experiments, potassium phosphate buffers containing 10% glycerol were used in the hydrophobic and hydroxyapatite columns, and a sodium phosphate buffer with 5% glycerol was used on the cation exchange column. These modifications resulted in improved stability of enzyme activity. Crystals of 15-lipoxygenase were grown by the vapor diffusion method [37]. The 15-lipoxygenase crystals obtained with and without glycerol in the purification buffers were similar in appearance.

RESULTS AND DISCUSSION

The final fraction of purified enzyme catalyzed the production of 9 µmol 13-hydroperoxy-octadecadienoic acid per min per ng protein (37°C), which is comparable to the specific activities reported elsewhere for purified rabbit reticulocyte lipoxygenase with linoleic acid as the substrate [17].

The four steps in the purification were evaluated by a 9% polyacrylamide-SDS gel (fig. 1A). The final sample contains a major band of 70,000 daltons which is immunoreactive to anti-15-lipoxygenase antibodies [38] on immunoblots (fig. 1B). Lower molecular weight bands appeared in various preparations but were also immunoreactive and were therefore thought to be degradation products of 15-lipoxygenase. Similar degradation of homogeneous protein has been noted for the human leukocyte 5-lipoxygenase [39]. The purity of the final fraction was estimated to be greater than 98%, with the predominant contaminant being the 65,000-dalton degradation product (fig. 1C). Using gel analysis to quantitate the amount of 15-lipoxygenase present in the initial ammonium sulfate step, we estimate the yield of the procedure to be approximately 8%. Approximately 8 mg of purified lipoxygenase can be obtained from 240 ml of rabbit blood; with repeated phlebotomy, over 15 mg of purified enzyme was obtained from a single animal.

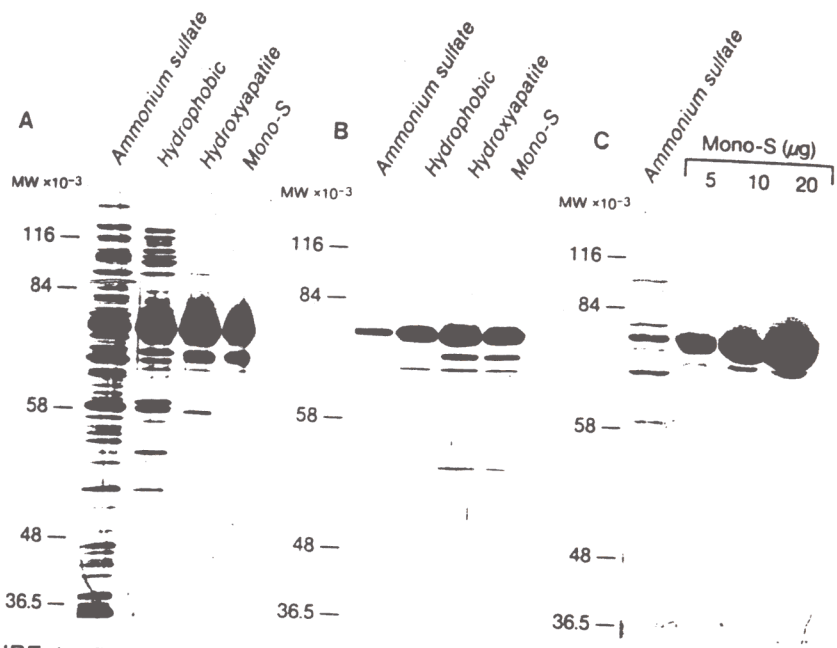

FIGURE 1. **Purification of 15-lipoxygenase from rabbit reticulocytes.** SDS-polyacrylamide gel analysis of protein samples from each purification step. A) Silver-stained gel. Protein from the peak fraction (2.5 μg) from each purification step was loaded as indicated. B) Western analysis. The immunoblot was developed with anti-human 15-lipoxygenase polyclonal antibodies and was visualized by goat anti-rabbit IgG conjugated with horseradish peroxidase [42]. C) Coomassie-stained gel. The ammonium sulfate fraction (20 μg) and increasing amounts of the final purified fraction are compared.

Crystals of 15-lipoxygenase were routinely grown by the vapor diffusion metho (both hanging and sitting drops) when the protein concentration was between 10 an 20 mg/ml. The crystals were shown to be 15-lipoxygenase by gel electrophoresis o dissolved crystals (data not shown). The largest crystals appeared within one wee when the drops were adjusted to a final concentration of 185 mM sodium phosphate pH 7.0, and the well solution was 375 mM sodium phosphate. The crystals were hexagonal rods (fig. 2, top). Crystals could be grown from protein which had beer purified through the hydrophobic interaction column. However, these crystals were too small to use, therefore the additional purification steps were taken to improve crysta growth. The largest crystals obtained reproducibly after the complete purificatior were, on average, 0.09 mm x 0.09 mm x 0.4 mm (fig. 2, bottom). The major facto affecting the growth of large crystals was the abundance of crystals in each drop Micro-seeding was attempted in an effort to increase the size of the crystals, but this resulted in showers of tiny crystals. To date, attempts to inhibit crystal nucleation with organic solvents and detergents have not been successful.

The crystals were shown to diffract using Cu K_α x-rays generated by a Rigaku rotating anode source operating at 3000 watts (50 kV, 60 mA). Still photographs showed that the crystals diffract to 5 Å, were slightly radiation sensitive, and were no twinned. Approximate unit cell dimensions (a = b = 260 Å, c = 145 Å) were obtained a

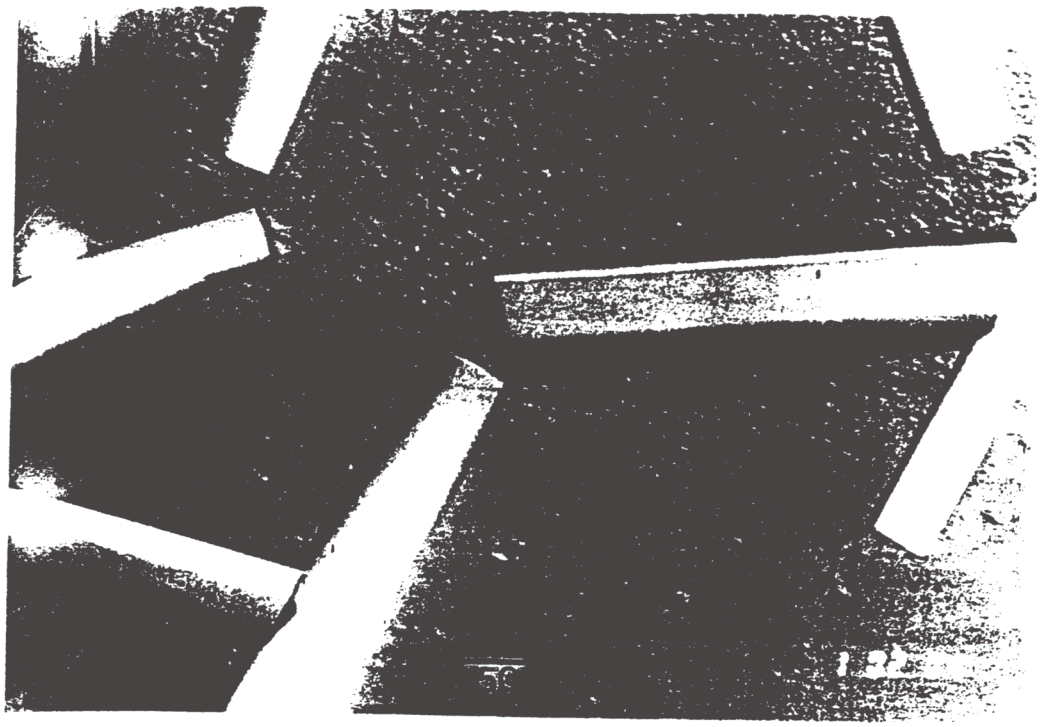

FIGURE 2. **Protein crystals of 15-lipoxygenase.** Crystals were grown by vapor diffusion in a hanging drop. The protein concentration in the drop was 7.5 mg/ml. The gradient for crystal formation was sodium phosphate, pH 7.0, 187-375 mM. The crystals routinely appeared within 5 days. Top) The hexagonal cross-section of the crystals shown is 0.05 mm. Bottom) The long dimension of the hexagonal rods was routinely 0.4 mm.

the EMBL Hamburg Outstation, using beam-line X11 on the DORIS storage ring. The cell was confirmed as hexagonal by the presence of a sixfold axis of symmetry in the crystal lattice, but the space group could not be determined unambiguously. Assuming a crystal volume/mass ratio of 2.4 $Å^3$ per dalton [40] and either 6 or 12 asymmetric units per unit cell, the asymmetric unit contains either an octamer or a tetramer, respectively. A search for other crystal forms of rabbit 15-lipoxygenase has produced crystals with the same morphology as those reported here.

Although the crystals of mammalian 15-lipoxygenase that we have obtained to date are suitable for low-resolution x-ray crystallographic analysis, we are also pursuing alternate crystal forms. Furthermore, the recent reports of various cloned mammalian lipoxygenases open up the possibility of using expressed lipoxygenase as a source for improving the crystal size or finding alternate crystals with more favorable symmetry and lattice constants. Towards this goal, we have expressed human 15-lipoxygenase in *E. coli* [41]. The expression of cloned 15-lipoxygenase in *E. coli* has also allowed us to generate site-specific mutants of 15-lipoxygenase to analyze the role specific amino acids play in the enzyme's activity. This information along with a three-dimensional structure will be essential for understanding the biochemical and biological function of lipoxygenases in various disease states.

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